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Original Research

Genotoxicity and cytotoxicity analysis of curcumin and sunset yellow in human lymphocyte culture

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Abstract: Genotoxic and cytotoxic effects of curcumin and sunset yellow were tested by the chromosome aberration analysis and cytokinesis-block micronucleus cytome assay in human lymphocyte culture. Water solutions of food dyes, in concentrations of 1, 2, 4 and 8 mM, were added to the cultures at the beginning of the cultivation period. Concentrations of 4 and 8 mM of sunset yellow induced significant increase in frequencies of cells with chromosome aberrations. Tested concentrations of sunset yellow significantly associated with frequencies of structural aberrations, chromatid-type aberrations, total aberrant cells and micronuclei showing considerable dose dependent clastogenic activity. In higher analyzed concentrations, curcumin significantly increased only nuclear buds frequency, suggesting its potential genotoxicity, while sunset yellow showed dose-dependent genotoxic potential. Obtained results point toward favorization of natural coloring agents in food consumption and emphasize the need of controlled use of food colorants.

Key words: Food dye; Chromosome aberration; Micronucleus; Nuclear bud.

Introduction

Effects of food coloration on the appetite and food attraction are extensively used in food technology (1, 2). However, modern researches have resulted in the notable reduction of the used food colorants (2, 3). Some of the synthetic food dyes are associated with the health and behavioral risks in children (4, 5, 6) and patients (7). Natural food pigments are generally desirable and often show protective (8) and antioxidative characteristics (9).

Genotoxicity and mutagenicity data of many food dyes are often inconsistent. Sunset yellow has been designated as genotoxic and cytotoxic in human lymphocytes (10,11), mice (12), root tip cells of *Allium cepa* L. (13) and *Brassica campestris* L. (14), but not mutagenic in *Ames* test (15) nor genotoxic in gut micronucleus assay in mice (16). There are also reports of sunset yellow contamination with carcinogens (17). Regardless the long medicinal and dietary use and increased popularity of turmeric, the powdered rhizome of *Curcuma longa*, as well as numerous proposed protective effects of curcumin, major ingredient of turmeric (18), higher applied concentrations of curcumin (50 µg/ml) increase chromosome aberrations (19) and micronuclei frequencies (20) in human lymphocytes *in vitro*. Curcumin has been proposed as an astonishing therapeutic with activities ranging from antiviral and anti-inflammatory to immunemodulating and anticarcinogenic or even efficient in treatment of complex diseases (18).

Given that available data regarding food colorants genotoxicity, although obtained from relevant tests and biomarkers, are often stunted and inconsistent, this study aimed to simultaneously evaluate and compare genotoxic effects of curcumin and sunset yellow using cytokinesis-block micronucleus cytome assay (CBMN-cyt assay) and chromosome aberration (CA) analysis in human lymphocytes *in vitro*. The significance of this study emphasizes the widespread use of food dyes and increased interest for consumption of organic products, including curcumin.

Materials and Methods

Food dyes

Sunset yellow (E-110) and curcumin (E-100), purchased in the powder form (Sigma-Aldrich Co., St. Louis, MO), were used for preparation of water solutions in concentrations of 1, 2, 4 and 8 mM and added into prepared growing medium for human lymphocyte cultures.

Cell cultures and applied assays

Four healthy participants donated peripheral blood samples, which were collected into sodium heparin vacutainers (BD Vacutainer Systems, Plymouth, UK). The participants, under the following criteria: no smoking, no exposure to medical ionizing radiation (for at least 3 months before the study), no chronic illnesses (including diabetes) and no consumption of antibiotics, signed informed consent forms. The Scientific Council of the Institute for Genetic Engineering and Biotechno-

logy approved and confirmed the study appropriateness to ethics standard.

Using PB-Max Karyotyping medium (Life technologies, Carlsbad, CA) independent culture replicates (for each food colorant, tested concentrations and control) were established for applied tests: chromosome aberration analysis and cytokinesis-block micronucleus cytome assay. Cultures were treated respectively with the 100 µl of the sunset yellow or curcumin water solutions (1, 2, 4 and 8 mM) and 100 µl of ddH2O for negative controls.

Metaphases, for chromosome aberration analysis, were harvested 90 minutes upon colcemid (Invitrogen, Carlsbad, CA) treatment (0.18 µg/ml). For the CBMN-Cyt assay, cytochalasin B addition in the final concentration of 4.5 µg/ml blocked cytokinesis. Following centrifugation, and supernatant removal, cells were treated with 0.56% KCl hypotonic solution, fixed with ethanol-acetic acid and dropped on chilled and coded slides. Air-dried slides were stained in 5% Giemsa (Life technologies, Carlsbad, CA). Per each replicate 200 metaphases were observed. CBMN-Cyt assay included observation of micronuclei, nuclear buds and nucleoplasmic bridges in 2000 binuclear cells and a total of 500 cells to calculate nuclear division indexes (NDI and NDCI) (21).

Statistical analysis

Upon Shapiro-Wilk assessment of distribution normality, ANOVA (one-way analysis of variance) followed by Newman-Keuls multiple comparisons was applied (MedCalc for Windows Software, version 16.2.0 Ostend, Belgium). The significance level was set at $p<0.05$. Relationships between parameters frequencies and applied concentrations were tested by simple linear regression (MedCalc 16.2.0).

Results

Structural aberrations were analyzed in metaphases containing 46±1 chromosomes and classified as chromatid-type (breaks and minutes) and chromosome-type aberrations (breaks, minutes and rearrangements e.g. dicentrics). The results of chromosome aberration analysis are gathered in table 1.

The most frequent aberrations in cultures treated with curcumin were chromatid breaks (Figure 1) and acentric fragments (Figure 2). No chromosome rearrangements were observed neither in curcumin nor in sunset yellow treated cultures. Curcumin did not significantly affect frequencies of observed chromosome aberrations (cht, chr and aberrant cell) in any of the tested concentration. Sunset yellow treatments were the most effective in induction of acentric fragments (Figure 3), followed by chromatid breaks (Figure 4). The observed increase in chromosome aberrations frequencies in 4 and 8 mM sunset yellow treatments has been significant exclusively for the frequencies of aberrant cells against negative control ($F=4.6$; $p=0.013$) (Table 1). Dose-dependent correlation was confirmed between sunset yellow concentrations and frequencies of chromatid-type

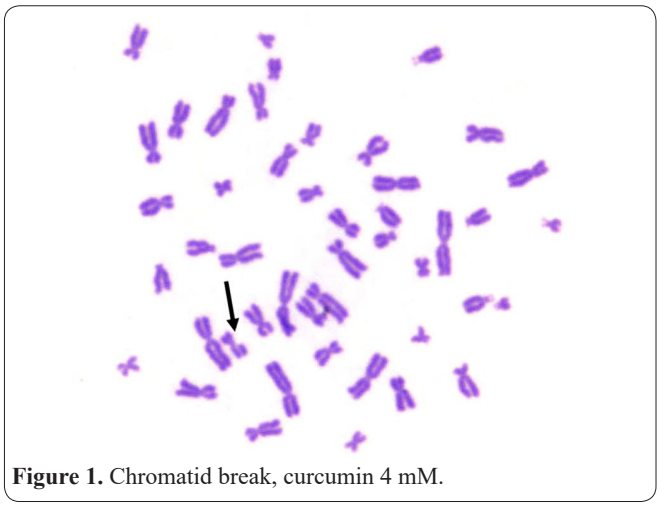

Figure 1. Chromatid break, curcumin 4 mM.

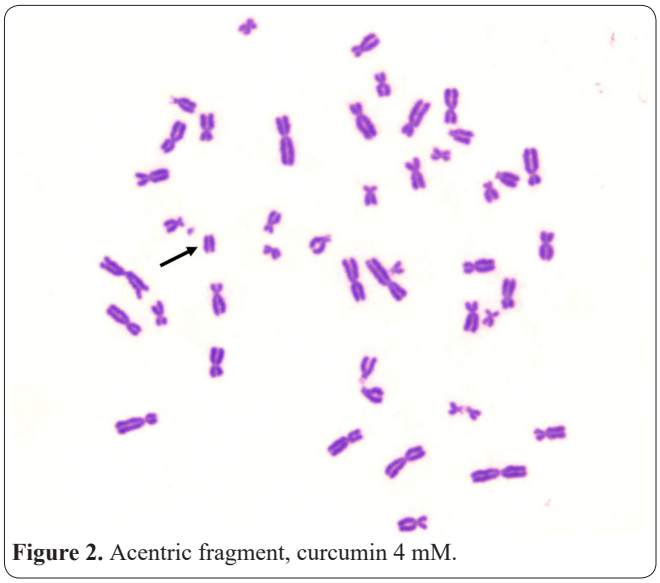

Figure 2. Acentric fragment, curcumin 4 mM.

Table 1. Chromosome aberrations in human lymphocytes treated with curcumin and sunset yellow.

Colorant	Treatment (N=4)	Type of aberration*			
		cht (a)	chr (b)	Structural aberrations Σ (a+b)	aberrant cells
Control		0.25±0.50	1.75±1.71	2.00±2.16	1.00±0.82
Curcumin	1 mM	1.00±1.41	1.00±0.82	2.00±2.00	2.00±2.00
	2 mM	1.75±1.50	0.75±0.50	2.50±1.29	2.50±1.29
	4 mM	2.00±1.41	1.75±1.71	3.75±2.99	3.25±2.06
	8 mM	1.00±0.82	1.50±0.58	2.50±1.29	2.50±1.29
Sunset yellow	1 mM	0.50±0.58	0.75±0.5	1.25±0.96	1.25±0.96
	2 mM	1.00±1.16	1.75±1.26	2.75±1.89	2.00±1.63
	4 mM	1.75±0.96	3.00±2.71	4.75±2.22	3.50±0.58[a]
	8 mM	2.00±1.63	2.00±1.41	4.00±1.83	3.50±1.29[a]

*in 4 replicates; 200 metaphases per each replicate - expressed as the mean±SD; cht - chromatid-type aberrations (a); chr – chromosome type aberrations (b); total structural aberrations Σ(a+b). [a]Significantly increased against negative control ($p<0.05$).

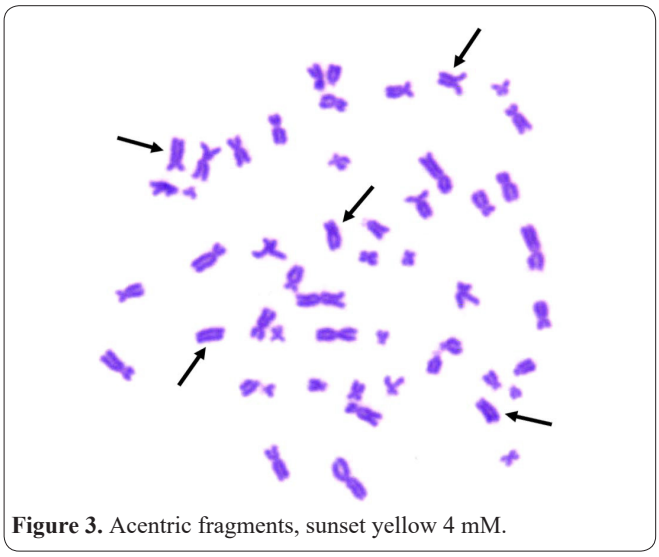

Figure 3. Acentric fragments, sunset yellow 4 mM.

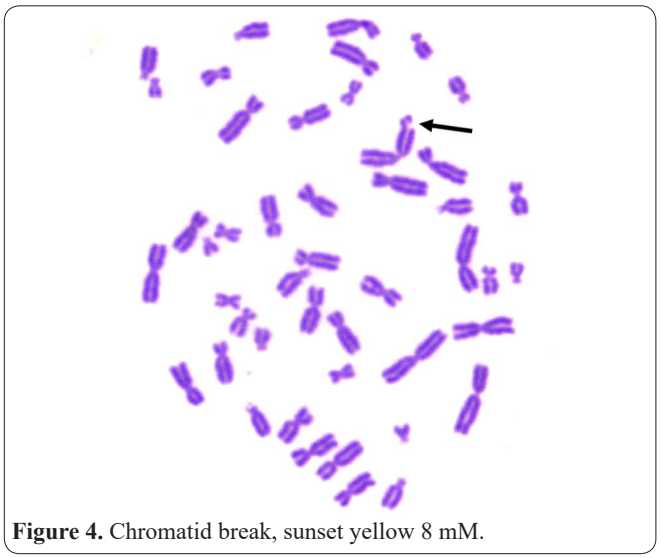

Figure 4. Chromatid break, sunset yellow 8 mM.

aberrations (p=0.01), structural aberrations (p=0.04) and aberrant cells (p=0.015). Effects of curcumin on chromosome aberrations induction were not dose related.

CBMN-cyt assay results are shown in table 2. Curcumin, in 4 and 8 mM treatments significantly increased frequency of nuclear buds against control and 1 mM treatment (F=6.761; p=0.003). The frequency of nuclear buds showed significant association with the applied concentrations of curcumin (p=0.006). Sunset yellow was not genotoxic nor cytotoxic in CBMN-cyt assay, although increase in applied concentrations of sunset yellow significantly correlated with the increase in micronuclei frequencies (p=0.008).

Discussion

Although numerous studies regarding curcumin and sunset yellow genotoxicity have been previously performed (10, 19, 20), none of them combined chromosome aberrations analysis and CBMN-cyt assay nor simultaneously evaluated curcumin and sunset yellow effects in the same conditions *in vitro*. Concentrations of curcumin and sunset yellow, tested in this research (1-8 mM), correspond to 7-56 μg/ml and 8-64 μg/ml respectively. The tested concentrations were set according to the study of Sebastià *et al*. (19), since concentrations of curcumin, higher than 50 μg/ml, are reported to be genotoxic to human lymphocytes (19), as well as higher (10-40 mg/ml), previously tested concentrations of sunset yellow (10).

Acceptable daily intake (ADI) level for curcumin is 3 mg/kg/bw/day (22). Reported levels of maximum exposure to the sunset yellow are generally below the 1 mg/kg/bw/day, although it could be higher in children. The safety of sunset yellow was re-evaluated and in 2009 EFSA set a temporary ADI of 1 mg/kg/ bw/day (from previous of 2.5 mg/kg/bw/day) with the recommendation for further tests to be conducted (23). Estimating that consumers´ exposure to sunset yellow is well below in 2014 EFSA has established an ADI of 4 mg/kg bw/day for all age groups (24). However, sunset yellow is banned in Norway and Finland (25) as well as in the United States and Japan (26).

Despite protective and antioxidative properties, natural food colorants often lack stability, while adverse effects for some synthetic food colorants are proven (2, 12, 27-29). Concerns in food dyes consumption also come from proposed cumulative effects of food colorants used without proper evaluation (30) as well as from the numerous food products that contain food dyes and contribute to the total exposure to food colorants (24).

Therapeutical and protective properties make curcumin one of the most extensively studied naturally-derived products. However, both benefitial (18, 20) and

Table 2. CBMN-cyt assay of lymphocytes treated with curcumin and sunset yellow.

Colorant	Treatment (N=4)	Genotoxicity biomarkers*			Cytotoxicity indexes**	
		MNi	NBs	NPBs	NDI	NDCI
Control		19.75±6.19	5.25±2.63	2.00±1.41	1.48±0.28	1.47±0.27
Curcumin	1 mM	27.50±8.58	6.50±1.29	4.00±2.00	1.43±0.19	1.43±0.19
	2 mM	28.25±6.45	8.751.89	4.00±2.94	1.48±0.27	1.47±0.26
	4 mM	32.25±12.04	13.00±2.16 [a]	4.25±2.22	1.36±0.18	1.35±0.18
	8 mM	27.25±7.14	10.75±3.00 [a]	4.25±2.75	1.34±0.11	1.33±0.11
Sunset yellow	1 mM	29.25±9.22	9.50±2.65	3.75±2.22	1.38±0.20	1.38±0.20
	2 mM	33.25±4.57	8.755.68	6.25±4.99	1.39±0.19	1.38±0.18
	4 mM	35.25±10.01	10.00±4.08	6.75±3.30	1.46±0.18	1.46±0.18
	8 mM	40.50±13.63	10.75±2.75	7.25±6.13	1.44±0.22	1.43±0.22

*in 4 replicates, 2000 BN cell per each replicate; **500 analyzed cells per each replicate - expressed as the mean±SD; BN – binuclear cell; MNi – micronuclei; NBs – nuclear buds; NPBs – nucleoplasmic bridges; NDI – nuclear division index; NDCI – nuclear division cytotoxicity index.

[a] Significantly increased against negative control and 1 mM treatment (p<0.05).

undesirable effects are observed (18, 19). However, curcumin has also been identified as a PAINS (pan assay interference) compound (31) due to its interfering with readout in different assays and an IMPS (invalid metabolic panaceas) compound (32). That may be the cause for unproven beneficial effects of curcumin in numerous conducted clinical trials (18). Previous genotoxicity and cytotoxicity analysis of curcumin in concentrations ranging from 0 to 50 μg/ml have shown that higher concentrations induce chromosome aberrations, mainly acentric fragments, in human lymphocytes *in vitro* (19). The higher used concentrations of curcumin, from the range of 0.5 to 128 mg/ml, increase micronuclei frequency but also have significant effect on the reduction of micronuclei frequency induced by cisplatin in PC12 cells (20). Similar findings are found in HepG2 cells. In concentrations of 8 and 16 μg/ml curcumin significantly increases micronuclei frequency in HepG2 cells although lower concentrations (2 μg/ml) significantly reduce frequency of cyclophosphamide induced micronuclei (33) or parathion induced sister chromatid exchanges (34). The frequency of micronuclei, in human lymphocytes upon irradiation by ^{131}I significantly decreases after curcumin treatment in concentrations of 5, 10, and 50 μg/ml (35).

Differing from findings of Sebastia *et al.* (19), we did not observe remarkable increase in chromosome aberrations after curcumin treatment, even in the highest applied concentration of 8 mM (56 μg/ml). However, we revealed significant dose-dependent increase in frequency of nuclear buds. Nuclear buds are structures responsible for the expulsion of undesirable DNA content (36) and extra chromosomes from the cell (37) through recombination between homologous regions within amplified sequences forming acentric fragment or double minutes (38). As intermediate phase in micronuclei formation, nuclear buds present reliable markers of genotoxicity. This finding additionally points toward increased genotoxicity of higher curcumin concentrations. High SD values for genotoxicity biomarker in CBMN-cyt assay are evidenced in control but also cultures treated with curcumin and sunset yellow. Micronuclei and consequently their precursors, nuclear buds are highly influenced by demographic variables and/or dietary factors showing intra- and inter-individual variation with the SD reported to range from 3.3-17.7 in healthy population (39).

Additional concern regarding curcumin′s activity is its variable purity and chemical instability both *in vitro* and *in vivo*. Pharmacokinetic and pharmacological properties of curcumin, determined by available ADMET qualities (absorption, distribution, metabolism, excretion and toxicology) are rather not promising, especially regarding low absorption an bioavailability (40) and efforts to improve them were so far unsuccessful (18). However, further ADMET research is recommended for the compounds of curcumin mixture, following its characterization.

Frequency of observed genotoxicity and cytotoxicity parameters between sunset yellow treatments and negative control did not significantly differ according to ANOVA. However, simple linear regression showed significant linear associations between concentrations and following parameters: chromatid - type and structural aberrations, aberrant cells and micronuclei. Significant increase in micronuclei frequency has also been detected in human lymphocytes by Kus and Eroglu (10), although in higher applied concentrations (10, 20, 30 and 40 mg/ml), compared to those that we tested (equal to 8-64 μg/ml). In the research of Sayed and colleagues (12) it has been demonstrated that sunset yellow increases frequencies of sister chromatid exchanges and chromosome aberrations in mice, especially in repeated oral treatments. Nevertheless, selenium, in combination with A, C and E vitamins, significantly reduces genotoxic effects of sunset yellow. Sunset yellow is not genotoxic in the gut micronucleus assay in mice (16) nor in *Ames* test, with or without metabolic activation (15). Swaroop and colleagues (11) have found that genotoxicity of sunset yellow in human lymphocytes observed by cytokinesis-block micronucleus cytome assay was weaker in comparison with the other tested synthetic colorants or their combinations.

Despite the fact that dose-dependent clastogenic potential of sunset yellow has been determined as well as the increase in nuclear buds frequency in higher applied concentrations of curcumin, results of this work imply that those tested food colorants are not remarkable genotoxins in applied concentrations. However, the controlled use and continuous investigation, including additional ADMET analysis *in vivo*, of food colorants is the crucial to avoid undesirable effects and achieve meaningful, interpretable results. Those should be considered along with expanded use of curcumin due to its protective properties.

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Interest conflict
All authors declare that they have no conflict of interest associated with this publication. We confirm that the final version of the manuscript has been read and approved by all named authors listed in the agreed order.

Authors´ contribution
A.Haverić, S. Haverić and S. Ibrulj conceived and planned the experiments. A. Haveric, S. Haveric and M. Hadzic conducted experiments and together with N. Lojo-Kadric and S. Ibrulj performed statistical analysis and interpretations of results. A. Haveric wrote the manuscript.

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